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SYNERGISTIC TOXIC EFFECTS OF CITRININ, OCHRATOXIN A AND PENICILLIC ACID IN MICE

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G. A. SANSING, E. B. LILLEHOJ, R. W. DETROY and M. A. MILLER. Synergistic toxic effects of citrinin, ochratoxin A and penicillic acid in mice. *Toxicon* **14**, 213–220, 1976.—The LD_{50}'s in mice of citrinin, ochratoxin A, and penicillic acid injected intraperitoneally were 89, 22, and 100 mg per kg of body weight, respectively. Paired combinations of the mycotoxins, citrinin: ochratoxin A (CI:OA), ochratoxin A:penicillic acid (OA:PA), and penicillic acid:citrinin (PA:CI) elicited synergistic lethal responses. After administration of citrinin, ^{14}C-orotic acid incorporation into both liver and kidney increased significantly by 27 hr with a return to control levels at 51 hr. Treatment with penicillic acid also increased orotic acid incorporation into liver ribonucleic acid (RNA) at 27 hr and at 4 hr in the kidney. Ochratoxin A inhibited orotic acid incorporation into both liver and kidney RNA 6 hr after toxin injection with a subsequent return to control levels at 27 hr. Orotic acid incorporation was inhibited in both kidney and liver 6 hr after treatment with the toxin combination CI:OA. PA:CI stimulated precursor incorporation into kidney RNA at 27 hr and inhibited the function in liver. The OA:PA combination inhibited orotic acid incorporation in both organs 15–27 hr after toxin treatment.

INTRODUCTION

THE HAZARD associated with the natural occurrence of mycotoxins in foods and feeds is compounded by the simultaneous presence of two or more biologically active fungal metabolites; this danger is enhanced if the substances elicit a synergistic toxic response. Natural contamination of grains by citrinin, ochratoxin, or penicillic acid has been identified (KROGH *et al.*, 1973, 1974a, b,; SCOTT *et al.*, 1972; SHOTWELL *et al.*, 1971; THORPE and JOHNSON, 1974). The simultaneous occurrence of ochratoxin and citrinin in naturally contaminated commodities has also been observed (SCOTT *et al.*, 1972; KROGH *et al.*, 1973). Fungal strains have been isolated that concomitantly produce either ochratoxin and citrinin (CIEGLER *et al.*, 1973; KROGH *et al.*, 1973; SCOTT *et al.*, 1972) or ochratoxin and penicillic acid (CIEGLER, 1972). In addition, isolates of the fungus responsible for the contamination of grains with ochratoxin, *Penicillium viridicatum*, have been obtained that also produce citrinin and penicillic acid (SCOTT *et al.*, 1972; KROGH *et al.*, 1970).

Feeds contaminated with *P. viridicatum* have been related to a nephrotoxicity syndrome

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in swine (KROGH *et al.*, 1970) and kidney and liver damage in laboratory animals (BUDIARSO *et al.*, 1971; CARLTON and TUITE, 1970). Many pathological and clinical studies have been carried out on the effects of individual toxins produced by *P. viridicatum* in laboratory and domestic animals (CARLTON *et al.*, 1970, 1974; CIEGLER *et al.*, 1971; KROGH *et al.*, 1974a, b; SAITO *et al.*, 1971; STEYN, 1971; SZCZECH *et al.*, 1973a, b, c). Independently, citrinin, ochratoxin, and penicillic acid seem particularly toxic to kidney and liver cells. A preliminary study of ochratoxin and penicillic acid showed that mixtures of the two substances produced a synergistic lethal response in mice (LINDENFELSER *et al.*, 1973).

We investigated the toxicity of citrinin, ochratoxin, and penicillic acid further by determining the lethality in mice of combinations of these substances. We also determined how these toxins affected incorporation of ^{14}C-orotic acid into ribonucleic acid (RNA) of mouse liver and kidney. Mycotoxin-mediated changes in RNA metabolism have been attributed to a fundamental, toxic response in cells of target tissues (AKAO *et al.*, 1971; NEAL, 1972).

MATERIALS AND METHODS

Female mice (CD-1 outbred albino strain; 20–22 g body weight) were purchased from the Charles River Mouse Farms, Inc.,* Wilmington, Mass., and housed in a constant temperature room (25°C). Animals were fed a standard pelleted diet and given tap water *ad libitum*. All animals were raised to 25 g body weight before treatment.

All mycotoxins used were produced at the Northern Laboratory by the following methods: citrinin, SAITO *et al.* (1971); ochratoxin, NESHEIM (1969); and penicillic acid, KURTZMAN and CIEGLER (1970). The mycotoxins were purified by chromatographic and crystallization procedures; purity was defined by standards established for each toxin (SANSING *et al.*, 1974). ^{14}C-orotic acid, specific activity of 24·3 mCi per mM, came from Calatomic Inc., Los Angeles, Calif.

LD_{50}'s for the mycotoxins were determined by intraperitoneal (i.p.) injection of test animals (six/treatment) with solutions prepared in sterile, pyrogen-free saline adjusted to pH 8·2 with sodium bicarbonate. (A pH of 8·2 increased solubility of mycotoxins.) All dosages were adjusted to provide the required amount in 0·1 ml of injection carrier. Since almost all deaths occurred before 48 hr, and rarely after 72 hr, the LD_{50} determinations were based on mortality at 72 hr. LD_{50}'s were calculated by WEIL's (1952) method with the following constants: $n = 6$, $K + 1 = 4$, and $d = 0{\cdot}301$, where n = the number of animals dosed per treatment level; $K + 1$ = the number of dosage levels employed for each test substance; and $d = \log R$, where R is the geometric factor utilized for the difference between successive dose levels (in this study, $R = 2$).

Effects of the mycotoxins on kidney and liver function in mice were measured by the extent of ^{14}C-orotic acid incorporation into RNA of the organs. Labeled orotic acid was injected (i.p.) at 25 μCi per kg body weight at 1, 3, 12, 24, 48, or 72 hr after toxin injection (i.p.). Animals were initially treated with the following levels of individual toxins and combinations: (1) citrinin, 22 mg per kg; (2) ochratoxin A, 6 mg per kg; (3) penicillic acid, 25 mg per kg; (4) citrinin, 3 mg per kg-ochratoxin A, 3 mg per kg; (5) penicillic acid, 3 mg per kg-ochratoxin A, 3 mg per kg; (6) citrinin, 10 mg per kg-penicillic acid, 6 mg per kg. At various times after each treatment, the mice were injected with labeled orotic acid; 3 hr later they were sacrificed and macromolecular constituents of liver and kidney collected to determine the amount of radioactivity in RNA. Orotic acid and mycotoxin solutions were prepared with sterile, pyrogen-free saline (pH 7·2). All dosages were adjusted to provide the required amount of material in 0·1 ml of solution. Three hr after orotic acid injection, incorporation of the radioactive precursor into RNA was determined by a modification of the method described by BLOBEL and POTTER (1968). Eight mice were used per treatment time and their tissues assayed separately in order to determine variations between animals within an individual treatment.

Animals were sacrificed without anesthesia by stunning with a sharp blow to the head and subsequent decapitation. Livers and kidneys were removed immediately, weighed, and chilled in ice-cold 0·25 M sucrose in TKM buffer (TKM = 0·05 M Tris–HCl, pH 7·5, 0·025 M KCl, and 0·005 M $MgCl_2$). Individual livers and kidneys were homogenized in a Potter–Elvehjem apparatus in 3·0 ml and 2·0 ml of TKM buffer, respectively. The homogenate was centrifuged at 10,000 *g* for 20 min at 0°C. The supernatant fluid was decanted into cold test tubes with subsequent transfer of 0·1 ml to tubes containing 1·9 ml of 0·3 M perchloric acid at 0°C and thorough mixing of the two components. The acidified samples were incubated for 15 min at 0°C and the precipitated macromolecules collected by filtration on Whatman Grade GF/A glass fiber filters. The precipitates were washed with 2·0 ml of 0·3 M perchloric acid (0°C), air dried, and placed in

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

glass scintillation counting vials. After 10 ml of Bray's scintillation fluor (BRAY, 1960) was added to the test vials, radioactivity was measured in a Packard Tri-Carb (Model 2009) scintillation spectrometer at a counting efficiency of 49%.

RESULTS

In initial studies, the LD_{50} of citrinin was established as 89 mg per kg body weight, ochratoxin A at 22 mg per kg, and penicillic acid at 100 mg per kg. Subsequently, lethalities caused by simultaneous injections of mycotoxin combinations were determined (Table 1). No deaths occurred among the control animals.

LD_{50} values for the individual mycotoxins and the paired toxins were plotted as isobolograms (Fig. 1) according to the method described by HEWLETT (1969). The straight dashed line presents the expected LD_{50} of toxin combinations based on a simple additive

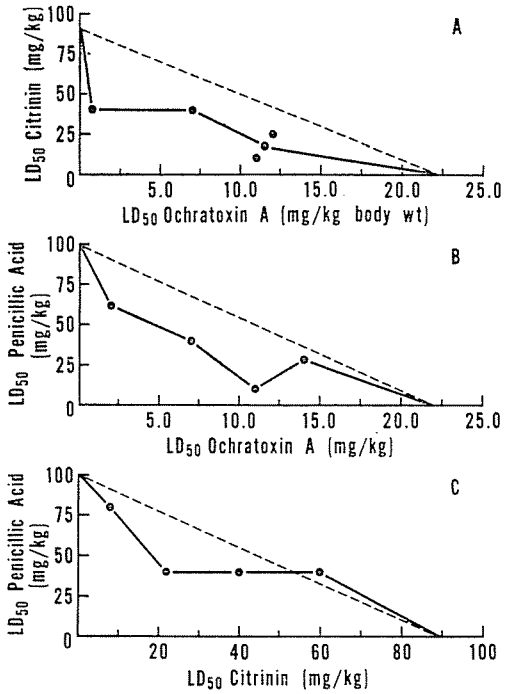

FIG. 1. ISOBOLOGRAMS OF LD_{50}'S IN MICE INJECTED WITH THREE COMBINATIONS OF CITRININ, OCHRATOXIN A, AND PENICILLIC ACID.
The dashed line represents expected LD_{50} of combinations assuming additive response. The solid line represents observed LD_{50} values.

response. Plots of actual values observed in mice treated with the three toxin combinations demonstrated synergistic toxic responses in all three cases. Calculations of the joint action ratio (R) (HEWLETT, 1969) for the toxin combinations resulted in the following values: $R = 2{\cdot}1$ for CI and OA; $R = 1{\cdot}7$ for OA and PA; and $R = 1{\cdot}6$ for PA and CI. These values indicate that all three combinations exhibited synergistic toxic responses with the citrinin–ochratoxin A combination resulting in the strongest effect.

Toxic properties of the substances was further examined by investigating their effects on how ^{14}C-orotic acid was incorporated into RNA of mouse kidney and liver. Citrinin slightly stimulated orotic acid incorporation into liver and kidney RNA early in the test

TABLE 1. LETHALITY (72 hr) IN MICE SIMULTANEOUSLY INJECTED WITH VARIOUS COMBINATIONS OF CITRININ (CI), OCHRATOXIN A (OA) AND PENICILLIC ACID (PA)

Dosages* (mg per kg)	Mortalities† (dead per total)	LD_{50}† (mg per kg)	Dosages (mg per kg)	Mortalities (dead per total)	LD_{50} (mg per kg)	Dosages (mg per kg)	Mortalities (dead per total)	LD_{50} (mg per kg)
CI:OA		CI:OA	OA:PA		OA:PA	PA:CI		PA:CI
200:0	6/6		50:0	6/6		200:0	6/6	
100:0	5/6	89:—	25:0	3/6	22:—	100:0	2/6	100:—
50:0	1/6	(61–113):(—)	12·5:0	1/6	(15–32):(—)	50:0	1/6	(69–140):(—)
25:0	0/6		6·2:0	0/6		25:0	0/6	
160:5	6/6		5:160	6/6		160:15	6/6	
80:2·5	6/6	40:1	2·5:80	2/6	2:64	80:7·5	1/6	80:8
40:1·3	2/6	(28–58):(0·8–1·7)	1·3:40	3/6	(1–3):(42–97)	40:3·8	1/6	(55–120):(5–11)
20:0·6	1/6		0·6:20	0/6		20:1·9	0/6	
80:15	6/6		15:80	6/6		80:45	6/6	
40:7·5	2/6	40:7	7·5:40	3/6	7:40	40:22·5	0/6	40:23
20:3·8	1/6	(28–58):(5–10)	3·8:20	0/6	(5–10):(29–55)	20:11·3	0/6	(29–55):(16–31)
10:1·9	0/6		1·9:10	0/6		10:5·6	0/6	
80:40	6/6		40:80	6/6		80:120	6/6	
40:20	6/6	25:13	20:40	3/6	14:28	40:60	0/6	40:60
20:10	1/6	(20–32):(10–16)	10:20	3/6	(9–22):(18–44)	20:30	0/6	(29–55):(44–82)
10:5	0/6		5:10	0/6		10:15	0/6	
22·5:22·5	5/6		22·5:22·5	5/6		100:100	6/6	
11·3:11·3	4/6	11:11	11·3:11·3	2/6	11:11	50:50	5/6	40:40
5·6:5·6	0/6	(7–17):(7–17)	5·6:5·6	0/6	(7–18):(7–18)	25:25	0/6	(32–50):(32–50)
2·8:2·8	0/6		2·8:2·8	0/6		12·5:12·5	0/6	

*Mycotoxins prepared with sterile, pyrogen-free saline adjusted to pH 8·2 with sodium bicarbonate. Toxin in 0·1 ml saline injected i.p.
†LD_{50} calculated from mortality data by the method of WEIL (1952).
Figures in parentheses represent 95% confidence intervals.

with a dramatic increase at 27 hr and a return to control levels at 51 and 75 hr (Fig. 2).

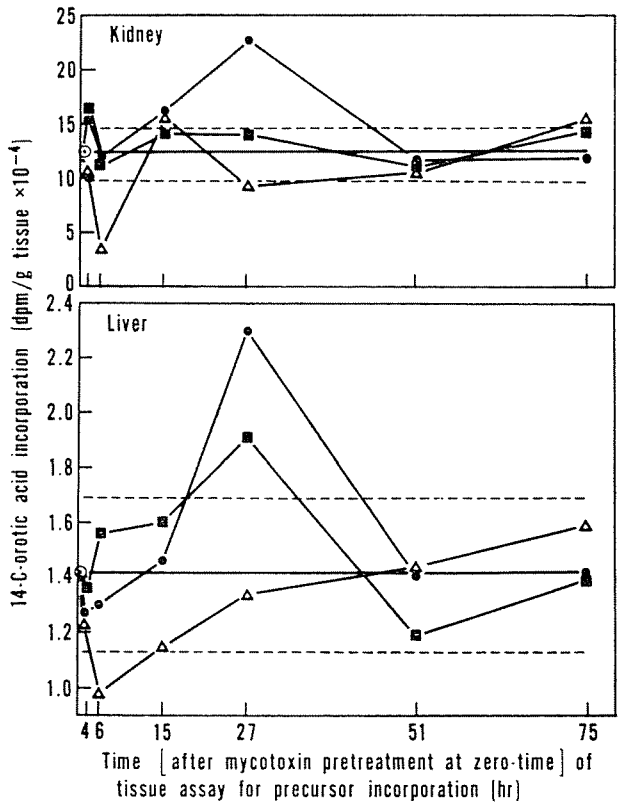

FIG. 2. TIME COURSE EFFECTS OF CITRININ (CI), OCHRATOXIN A (OA), AND PENICILLIC ACID (PA) ON ^{14}C-OROTIC ACID INCORPORATION INTO MOUSE KIDNEY AND LIVER RIBONUCLEIC ACID (RNA). Ordinate: incorporation of ^{14}C-orotic acid into RNA of kidney and liver tissue is expressed as dis. per min (dpm) per g. Mice (eight/toxin treatment/time interval) were pretreated with a mycotoxin (25% of the LD_{50} dose) at zero time (abscissa) and subsequently injected with radiolabeled orotic acid at 1, 3, 12, 24, 48 and 72 hr. ^{14}C-orotic acid (25 μCi per kg) was injected i.p. at the designated time). Kidney and liver samples were acquired from test animals 3 hr after orotic acid treatment. Treatments (mg per kg): CI (22) ●——●, OA (6) △——△, and PA (25) ■——■ were injected i.p. at zero time; (⊙—) represents the control animals treated with radiolabeled orotic acid at 1 hr and sacrificed 3 hr later for kidney and liver samples. The relative standard error of the mean based on the 8 control mice was 6·8%. Dashed line (- -) values were derived from addition or subtraction of the least significant difference (95% level) from controls. Values falling outside - - - are significantly different from controls.

Penicillic acid enhanced orotic incorporation slightly in kidney at 4 hr; a similar increase was observed in liver 27 hr after treatment with that toxin. Ochratoxin A inhibited incorporation in both tissues during the initial 6 hr after mycotoxin treatment with a return to control values in liver later in the test. After initial inhibition of incorporation in kidneys of ochratoxin-treated mice, the rate of orotic acid uptake increased at 15 hr with a subsequent decline at 27 hr and a return-to-control value at 51 hr.

Mycotoxin combinations affect orotic acid incorporation differently (Fig. 3). Initially, citrinin–ochratoxin A treatment stimulated orotic acid incorporation into kidney RNA

but in later stages generally inhibited it, whereas a significant inhibition was observed in liver uptake during the first 4–6 hr after treatment; a return-to-control levels, at 15 hr;

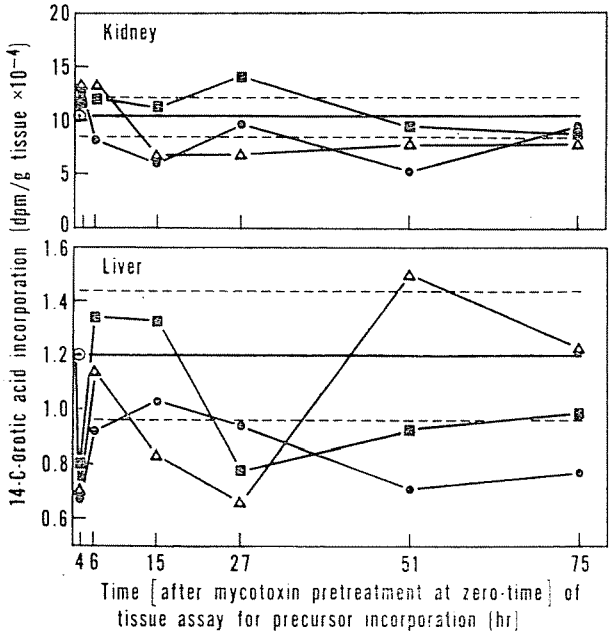

FIG. 3. TIME COURSE EFFECTS OF PAIRED COMBINATIONS OF CI, OA, AND PA ON ^{14}C-OROTIC ACID INCORPORATION INTO MOUSE KIDNEY AND LIVER RNA.

Ordinate: incorporation of ^{14}C-orotic acid into RNA of kidney and liver tissue is measured as dis. per min (dpm) per g. Mice (eight/toxin treatment/time interval) were pretreated with a paired combination of mycotoxins at zero time (abscissa) and subsequently injected with radiolabeled orotic acid at 1, 3, 12, 24, 48, and 72 hr. ^{14}C-orotic acid (25 μCi per kg) was injected i.p. at the designated times. Kidney and liver samples were acquired from test animals 3 hr after orotic acid treatment. Treatments (mg per kg): CI:OA (3:3) ●——●, OA:PA (3:3) △——△, and PA:CI (10:6) ▣——▣, were injected i.p. at zero time; (⊙—) represents the control animals treated with radiolabeled orotic acid at 1 hr and sacrificed 3 hr later for kidney and liver samples. The relative standard error of the mean based on the 8 control mice was 6·8%. Dashed line (- -) values were derived from addition or subtraction of the least significant difference (95% level) from controls. Values falling outside - - - are significantly different from controls.

and continued inhibition during the remainder of the test. The effect of PA:CI on incorporation into kidney RNA resembled the activity observed with the toxic substances administered independently; i.e. incorporation was stimulated 27 hr after mycotoxin treatment. However, the PA:CI combination inhibited incorporation into liver RNA at 27 hr with subsequent return to control levels at 75 hr. Ochratoxin A–penicillic acid treatment stimulated kidney incorporation at 4–6 hr with a reduction in uptake at the later stages of the test. Incorporation of labeled orotic acid in the liver of OA:PA treated animals was also inhibited at 4 hr. However, orotic acid incorporation by liver RNA in animals treated with OA:PA returned to control levels at 6 hr before extension of the inhibition at 15 and 27 hr and return-to-control values at 75 hr.

DISCUSSION

Our initial investigation of the acute toxicities of citrinin, penicillic acid, and ochratoxin

A in mice demonstrated that combinations of these mycotoxins elicited a synergistic response. Biochemical effects of toxin action were carried out further by determining how either the individual substances or the toxin combinations affected orotic acid incorporation into mouse kidney and liver RNA. Enhanced levels of RNA synthesis have been observed in liver after injury to the organ (CHAUDHURI *et al.*, 1967) and in other tissues treated with mitogenic stimulants (HENNINGS and BOUTWELL, 1970). In addition, a correlation has been established between early inhibition of orotic acid incorporation into RNA of target organs and subsequent tissue damage (AKAO *et al.*, 1971; NEAL, 1972).

The increased orotic acid incorporation in the kidneys of mice treated with citrinin and a combination of citrinin and penicillic acid suggests that a regenerative process was elicited after initial mycotoxin-induced damage. Apparently citrinin-mediated nephrotoxicity can be characterized by a delayed (27 hr) stimulation in orotic acid incorporation. In contrast to the increased level radiolabeled precursor accumulation observed in the RNA of kidneys and livers of mice treated with citrinin and penicillic acid, ochratoxin A distinctly inhibited orotic acid incorporation in both organs. The degree of interference with precursor incorporation elicited by ochratoxin in either kidney or liver suggests that this mycotoxin exhibits both nephrotoxic and hepatotoxic properties in mice. A similar type of suppression of orotic acid incorporation into RNA has been observed by WOGAN (1973) in liver of rats treated with aflatoxin B_1 and by AKAO *et al.* (1971) in mouse kidney after aflatoxin treatment.

Although penicillic acid clearly stimulated orotic acid incorporation into liver RNA 27 hr after treatment, the effects of penicillic acid combinations with citrinin or ochratoxin A did not resemble the pattern of enhancement elicited by penicillic acid alone. In fact, the PA:CI treatment inhibited precursor incorporation in liver during the initial 4 hr after treatment. Even though neither penicillic acid nor citrinin alone exhibited an inhibitory effect, this toxin combination gave a toxic response. The unique toxic manifestation observed in organs of mice treated with two toxins, plus the broad acute toxicity synergisms expressed by toxin pairs, emphasizes the concern about commodities simultaneously contaminated with two or more mycotoxins.

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